DISC ELECTROPHORESIS IN PLASMA PROTEIN ANALYSIS

A THESIS

SUBMITTED TO THE FACULTY OF ATLANTA UNIVERSITY IN

PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR

THE DEGREE OF MASTER OF SCIENCE

BY

EDITH YOUNG BIGGERS

DEPARTMENT OF BIOLOGY

ATLANTA, GEORGIA

AUGUST 1963

2a
36T

TABLE OF CONTENTS

Chapter		Page
I.	INTRODUCTION	1
II.	REVIEW OF LITERATURE	3
III.	MATERIALS AND METHODS	7
IV.	EXPERIMENTAL RESULTS	11
V.	DISCUSSION	13
VI.	SUMMARY AND CONCLUSIONS	14
LITERATURE CITED		15

LIST OF FIGURES

Figure | Page

1. The Disc Electrophoresis Trial Kit and Accessories..... 16

2. A Photograph of Rabbit Plasma Which Contained 10 Components Obtained by Disc Electrophoresis..... 17

3. A Photograph of Rabbit Plasma Which Contained 11 Components Obtained by Disc Electrophoresis..... 17

4. A Graph Showing a Pattern Which Contained 5 Components in Rabbit Plasma Obtained by Filter-paper Electrophoresis..... 18

5. A Photograph of Opossum Plasma Which Contained 4 Components Obtained by Disc Electrophoresis..... 19

6. A Graph Showing a Pattern Which Contained 4 Components in Opossum Plasma Obtained by Filter-paper Electrophoresis..... 20

CHAPTER I

INTRODUCTION

Electrophoresis may be defined as the migration of charged particles under the influence of an electric field. The speed and direction of movement of the particles depend upon the free charges on the surfaces of the particles. The migration of particles in an electric field was first observed in 1807 by Reuss, when a mixture of clay and water was subjected to an electric field and the clay particles migrated toward the anode.

Hardy (1899) demonstrated that negatively charged particles of denatured egg albumin could be made positive by the addition of an acid. With a change from alkaline to acid, the particles migrated in the electric field in the opposite direction. Therefore, the electrical characteristics of protein particles are conferred upon them by the nature of the medium in which the particles are dispersed.

Some particles when suspended in a solvent, will either be positively or negatively charged depending upon the nature of the particle. Proteins may be charged both positively and negatively, and are electrically neutral at their isoelectric point or isoelectric pH. At the isoelectric pH, proteins exhibit unique properties; that is, their solubility, hydration, and conductivity are at a minimum. The pH of a medium determines the dissociation of the acidic and basic groups, and greatly influences the net charges on a protein molecule. At a pH lower than the isoelectric pH a protein exhibits a net positive charge and migrates toward the cathode. The reverse is evident at a pH higher than the isoelectric pH.

The migration of particles in an electric field allows the separation of various components from a mixed protein solution. A protein solution, such

(Explanation of Figures)

Fig. 6. A graph showing a pattern which contained 4 components in opossum plasma obtained by filter-paper electrophoresis.

PLATE V

(Explanation of Figures)*

* All figures are photographs.

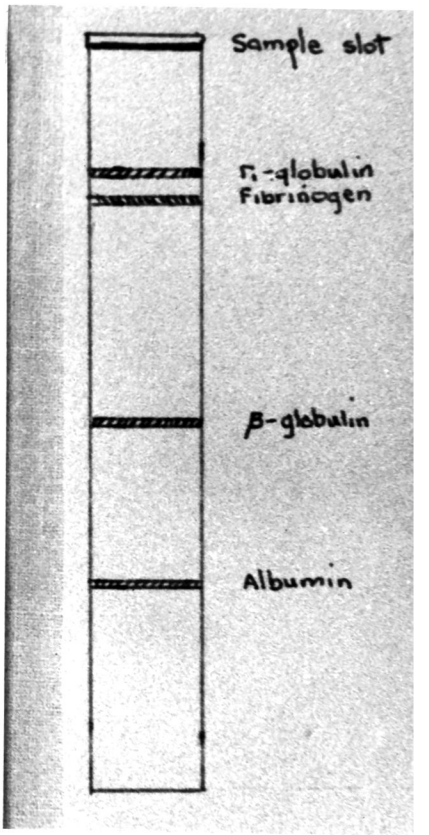

Sample slot
γ-globulin
Fibrinogen
β-globulin
Albumin

PLATE IV

(Explanation of Figures)*

*All figures are photographs.

(Explanation of Figures)

Fig. 5. A photograph of opossum plasma which contained 4 components obtained by disc electrophoresis.

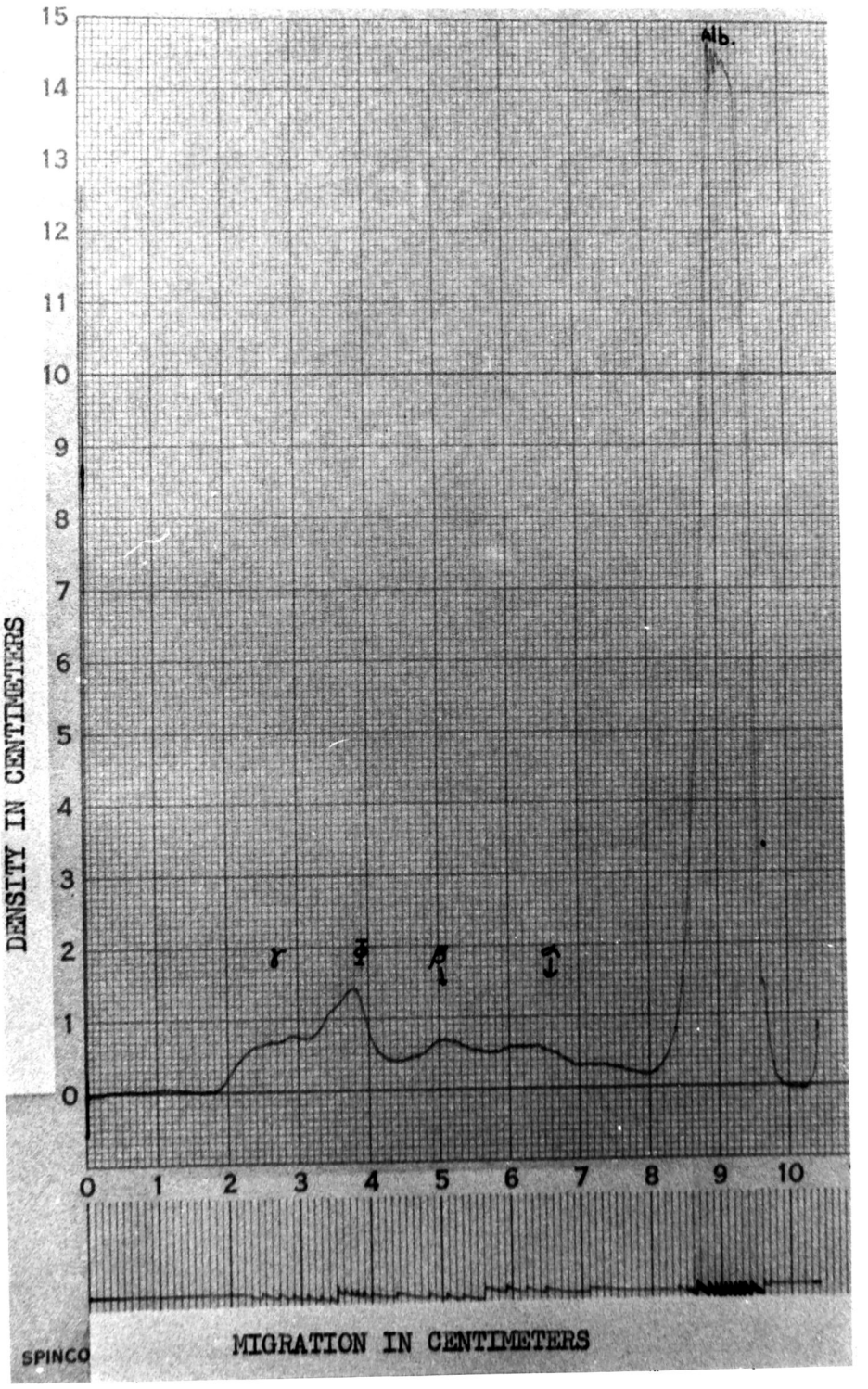

15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
Alb.
DENSITY IN CENTIMETERS
0 1 2 3 4 5 6 7 8 9 10
MIGRATION IN CENTIMETERS
SPINCO

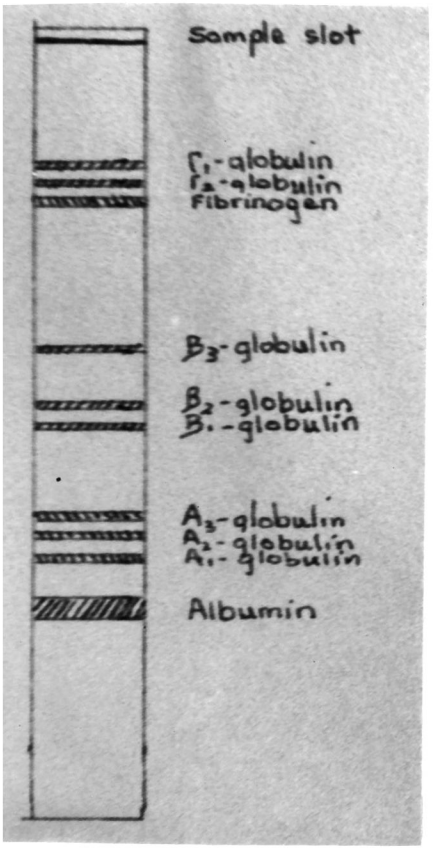
PLATE III

(Explanation of Figures)*

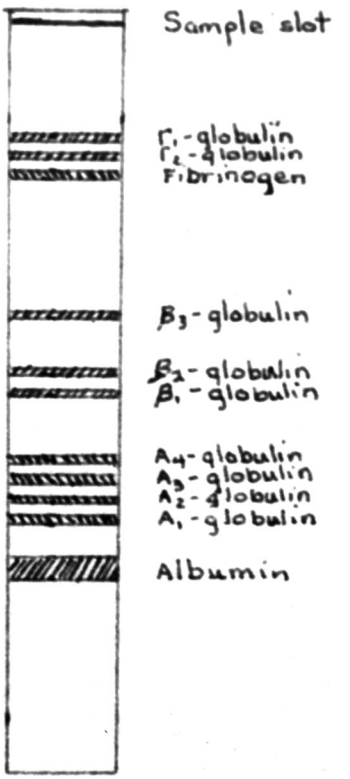
*
All figures are photographs.

(Explanation of Figures)

Fig. 4. A graph showing a pattern which contained 5 components in rabbit plasma obtained by filter-paper electrophoresis.


Sample slot
Γ_1-globulin
Γ_2-globulin
Fibrinogen
B_3-globulin
B_2-globulin
B_1-globulin
A_3-globulin
A_2-globulin
A_1-globulin
Albumin

2


Sample slot
Γ_1-globulin
Γ_2-globulin
Fibrinogen
B_3-globulin
B_2-globulin
B_1-globulin
A_4-globulin
A_3-globulin
A_2-globulin
A_1-globulin
Albumin

3

PLATE II

(Explanation of Figures)*

*All figures are photographs

(Explanation of Figures)

Fig. 2. A photograph of rabbit plasma which contained 10 components obtained by disc electrophoresis.

Fig. 3. A photograph of rabbit plasma which contained 11 components obtained by disc electrophoresis.

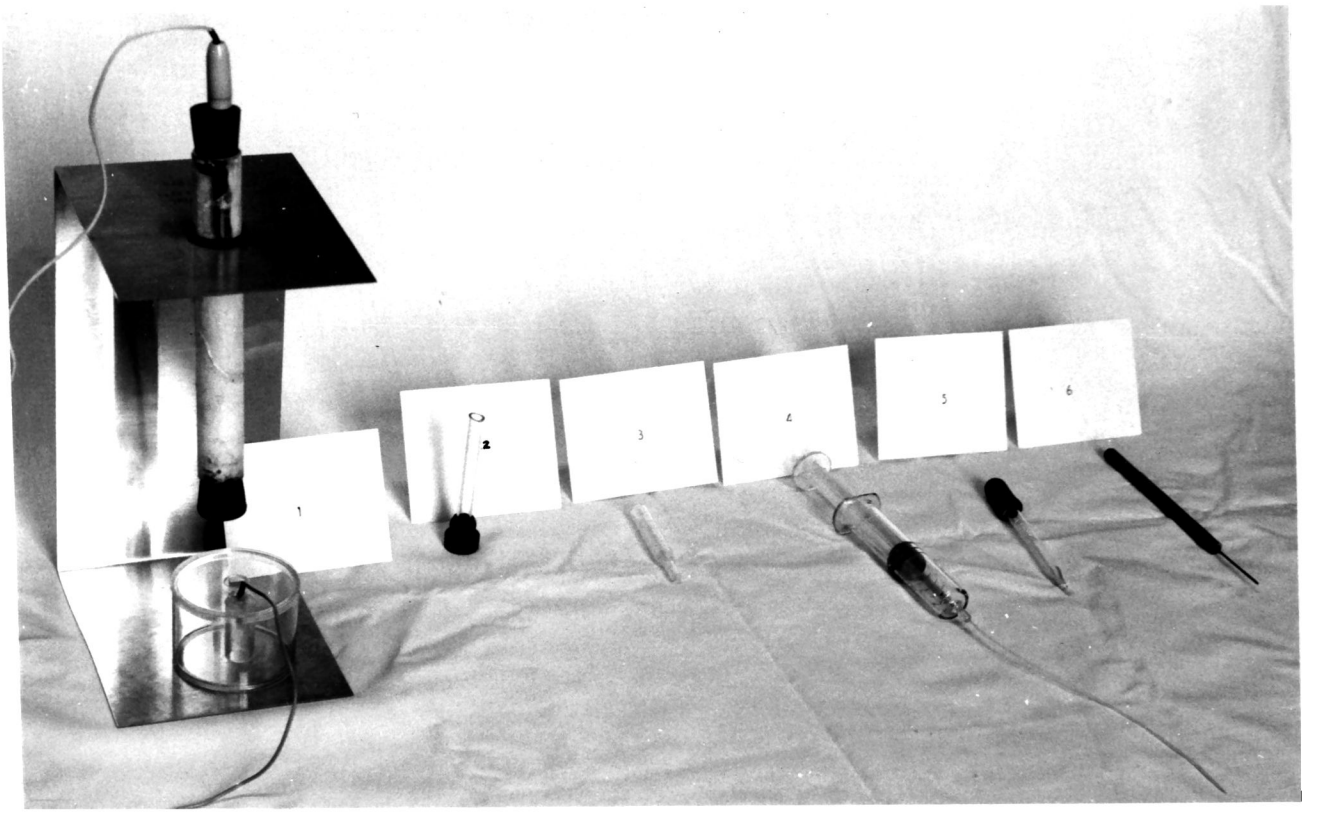

1
2
3
4
5
6

PLATE I

(Explanation of Figures)*

* All figures are photographs.

(Explanation of Figures)

Fig. 1. The disc electrophoresis trial kit and accessories; (1) stand, electrodes and buffer baths, (2) sample tube and base cap, (3) destaining tube, (4) syringe and capillary, (5) layering dropper and (6) removing tool.

as plasma has been separated electrophorectically into fibrinogen, globulins (alpha, beta, gamma), and albumin. Recent advances using serum proteins have indicated generic differences in plasmas from humans as well as that from other animals. The purpose of this investigation was to observe the pattern of plasma proteins in a polyacrylamide gel and determine if generic differences could be shown in the plasma proteins of rabbits and opossums.

CHAPTER II

REVIEW OF LITERATURE

Proteins move with different velocities in an electric field, to either the positive or to the negative pole, depending upon their net charges. Tiselius ('37) used the schlieren opitcal system for the construction of an electrophoretic (moving boundary method) apparatus. He identified albumin, beta and gamma globulins. With improvement of the Tiselius apparatus and by varying the pH of the buffer solution, other workers observed additional components found in the globulins, namely; $alpha_1$-, $alpha_2$-, $beta_1$-, $beta_2$-, and gamma globulins.

Svensson ('41) observed the sera of the horse, cow, swine and rabbit, using a phosphate buffer of pH 7.7 with an ionic strength of 0.2. According to the peaks, there appeared to be generic differences in the sera. There were 4 components identified in rabbit serum. Svensson also observed in the rabbit serum a higher percentage of albumin as compared with that of alpha, beta, and gamma globulins.

Deutsch and Goodloe ('45) separated rabbit plasma electrophorectically using a barbiturate buffer with a pH of 8.6. They observed 5 separable components. The amount of alpha-globulin was very small. However, a high content of albumin was present which resulted in an albumin-globulin ratio above that of man.

Moore ('45) observed sera from a variety of animals. There appeared to be a distinct pattern for each species. This might suggest generic differences in the plasmas of various animals. Moore further demonstrated that by varying the buffer solution, additional components could be observed.

Smithies ('55) introduced a zone electrophoresis method using a starch-gel. The starch-gel was used as the supporting medium, and the samples were placed on filter paper in a narrow zone. Electrical connections were made to the gels by filter paper wads soaked in buffer solution with the same pH as the gel. By the use of a regulated power supply, the pH of the gel remained constant throughout electrophoresis. When starch-gel was used as the supporting medium, the resolving power was superior to that of the Tiselius method.

Poulik and Smithies ('58) used human sera to correlate the number of separable proteins by a two-dimensional technique. The sera were placed on filter paper as described previously. After electrophoresis was carried out in a barbiturate buffer of pH 8.6, serial sections were cut from the filter paper and used for starch-gel electrophoresis. Each section was introduced into a transverse slit alongside the controlled filter paper soaked in the original serum sample. After the current was applied the protein components appeared as uninterrupted bands across the width of the gel.

The two-dimensional electrophoresis was used to validate the number of components, first, by the filter-paper method, and secondly, by the starch-gel method. In the separation of human sera, using the two-dimensional technique, more than 20 components have been demonstrated.

Smithies ('59) improved the resolution of protein components by preventing electro-decantation from occurring during the entry of migrating proteins into the gel, by carrying out electrophoresis with the gel in a vertical position. The samples were introduced directly into the slots of the starch-gel.

Sera from 6 healthy persons were examined, which included identical twins, a mother and three of her quadruplets. Two samples were taken from identical twins. The haptoglobin (Hp) and beta-globulin types from these twins were

the same. The resolution of the type 2-2 Hp in the sera revealed as many as 12 zones. The high-molecular weight of beta-lipoprotein showed as a clear zone between the slow alpha-globulin and the sample slot. However, the post-albumin as well as other separable proteins from the sera of the twins were the same.

Sera taken from three of the quadruplets were compared with sera taken from the identical twins. The Hp types of two of the quadruplets differed and were called Hp 2-2 and Hp 2-1. The post-albumins of these two individuals were the same, but differed from that of the identical twins.

The Hp type taken from the serum of the mother of the quadruplets was type Hp 2-1 but her post-albumin was different from that of the children. This suggested that genetic factors were involved in the post-albumin of normal individuals.

McArthur ('62) separated rabbit plasma into 5 fractions by the cold-ethanol technique. The precipitates, as well as the whole plasma, were analyzed on filter-paper and starch-gel.

In filter-paper electrophoresis, using a veronal buffer of pH 8.6, usually 5 components were demonstrated. However, the number of plasma proteins varied from 6 to 8. The separations were observed in order of decreasing mobilities as albumin, alpha, and beta globulins, fibrinogen and gamma globulin. The number of components was increased to 10, using the starch-gel technique. McArthur concluded that a complete isolation of proteins had not been accomplished, although components found in the starch-gel did not appear in paper electrophoresis. She further concluded that albumin migrated with the greatest mobility toward the anode and gamma globulin migrated in a retrograde direction toward the cathode, in the starch-gel.

Ornstein ('62) examined sera from humans, using a 7.5 per cent polyacrylamide gel. As many as 20 to 30 serum proteins were observed. The components were separated in fine layers 0.1 mm. or more thick, which in a column, resembled a stack of discs. The technique was called Disc Electrophoresis.

Prior to separation of the components, Disc Electrophoresis has been designed to take advantage of the adjustability of the pore size of a synthetic gel. This technique was found to be about 100 times more sensitive than the routine starch-gel technique. The protein components were resolved from volumes of whole human plasma as small as one microliter in a twenty minute run.

CHAPTER III

MATERIALS AND METHODS

The plasma used in this investigation was obtained from domestic rabbits (Sylvilagus floridanus) and opossums (Didelphis marsupialis). At least 6 ml. of blood were collected from each animal by intracardiac puncture and drawn into double oxalated syringes. This oxalate solution was prepared by adding 1.2 gm. of ammonium oxalate and 0.8 gm. of potassium oxalate to 100 ml. of distilled water. The tubes and hypodermic syringes contained 0.6 ml. of a 0.1 per cent oxalate solution. These tubes were dried overnight at 80°C, and cooled in an ice bath before using. The blood was centrifuged at 2000 rpm for 15 minutes. The plasma was removed by pipettes and refrigerated.

The electrophoresis apparatus used in this study was the Disc Electrophoresis Trial Kit, as illustrated in Figure 1. A Beckman Spinco Duostat was used as the power supply. The regulated power supply maintained a current of 5 ma. for electrophoresis.

Disc electrophoresis was carried out in columns of polyacrylamide which were divided into three sections; (1) a large-pore anti-convectional gel which contained the protein sample; (2) a large-pore gel in which protein concentration took place; and (3) a small-pore gel in which electrophoretic separation was accomplished.

The gel solution was loaded into open-ended cylindrical sample tubes which were 5 mm. in diameter and 70 mm. long. The base of the sample tube was stoppered and filled with 2 ml. of small-pore gel solution. This solution was carefully overlaid with 0.1 ml. of distilled water and allowed to polymerize for 40 minutes. The water layer was removed and the gel column rinsed with large-pore gel solution, after which 2 ml. of this solution were added

and carefully overlaid with distilled water. Sample tubes were placed about 3 inches below a fluorescent lamp. In 20 minutes the large-pore gel solution had photopolymerized. The water layer was removed, and 3 ul. of plasma were mixed with another large-pore gel solution and added. The sample was photopolymerized for 20 minutes.

An electrophoretic stand was placed in an upright position with an upper buffer bath suspended by an "O" ring. The stopper or base cap was removed from the sample tube, and a hanging drop of buffer (concentrated) solution was placed on both ends of the gel column, which prevented the formation of air bubbles during electrophoresis. The sample end of the tube was inserted into a silicone stopper until it was flush with the tapered end. The stopper and sample tube were fitted into the base of the upper buffer bath and the upper buffer tank was filled one inch from the top with concentrated buffer solution. The top was closed with a neoprene stopper which contained the electrode (cathode) and connected by a lead wire to the power supply.

A second buffer tank was placed on the base of the stand and filled one inch from the top with a dilute buffer solution. The electrode (anode) was located near the top of the lower bath. This bath was positioned so that the bottom of the sample tube passed through a hole in the top and extended 3/8 of an inch beneath the surface of the buffer solution. This electrode was then connected to the power supply. A current of 5 ma. was applied for 30 minutes.

Within 7 minutes after the current was applied a thin blue band of tracking dye appeared. This located the "front" which moved through the large-pore gels. Upon entering the small-pore gel, this dye component separated into two components. The leading thin blue line was the free tracking dye,

followed by a much thicker band, an albumin-dye complex. Electrophoresis was completed when the front migrated 1.25 inches into the small-pore gel. A removing tool and tap water (lubricant) were used to remove gels from the sample tubes. The gel was transferred into a glass holding tube for staining and destaining.

When staining was finished, usually one hour, the gel column was rinsed with tap water. A 7.5 per cent acetic acid solution was used for destaining. A current of 10 ma. was applied for one hour. After the gel which contained the separated proteins was cleared of unbound dye, destaining had been completed. The current was turned off and the gel was transferred into a glass tube which contained 7.5 per cent acetic solution for scanning or for storage.

The following stock solutions were necessary to prepare the small-pore and large-pore gel solutions.

A.	N Potassium hydroxide	48 ml.
	Acetic acid (glacial)	17.2 ml.
	Tetramethylethylenediamine	4.0 ml.
	Water to make	100 ml.
B.	N Potassium hydroxide	48 ml.
	Acetic acid (glacial)	2.87 ml.
	Tetramethylethylenediamine	0.46 ml.
	Water to make	100 ml.
C.	Acrylamide	60 gm.
	Methylene bis-acrylamide	0.4 gm.
	Water to make	100 ml.
D.	Acrylamide	10 gm.
	Methylene bis-acrylamide	2.5 gm.
	Water to make	100 ml.
E.	Riboflavin	4.0 gm.
	Water to make	100 ml.

The small-pore solution of pH 4.3 consisted of 1 part A, 2 parts B, and 1 part water. This solution was mixed prior to use with an equal volume of freshly prepared solution of ammonium persulphate (0.28 gm./100 ml.), which resulted in a solution containing 15 per cent acrylamide. Whereas, the large-pore solution of pH 6.8 consisted of 1 part B, 2 parts D, 1 part E, and 4 parts water.

The upper buffer of pH 6.8, referred to as concentrated buffer solution, was prepared as follows: Tris-hydroxy-methyl aminomethane 6.0 gm., glycine 28.8 gm., and water to make one liter.

Filter-paper electrophoresis was carried out using a Durrum-type electrophoretic cell from the Spinco Division of Beckman Instruments. The paper (Schleicher and Schuell 2043-A) was suspended on glass rods which served as a central support. These strips were saturated with a veronal buffer of pH 8.6 and allowed to drain for 15 minutes. An applicator was used to place the plasma on the center of the filter paper. A current of 2.5 ma. was applied for 17 hours.

After electrophoresis, one strip was removed, stained with bromphenol blue, and used as a guide in cutting out that portion of the unstained strips which contained specific plasma proteins. Each section was cut, while wet with the buffer solution, and placed in test tubes. Each segment of the paper strip was eluted with a 0.85 per cent solution of sodium chloride and then subjected to disc electrophoresis.

CHAPTER IV

EXPERIMENTAL RESULTS

Analyses were made on the plasmas of 11 rabbits and 5 opossums, using the disc electrophoretic technique. A buffer of pH 6.8 containing Tris-hydroxy-methyl aminomethane, glycine and water was used which resulted in the separation of rabbit plasma into 10 to 11 components.

The components were named in order of decreasing mobilities; albumin, $alpha_1$-, $alpha_2$-, $alpha_3$-, $alpha_4$-, $beta_1$-, $beta_2$-, fibrinogen, $gamma_1$-, and $gamma_2$ globulins, as illustrated in Figure 2.

Albumin migrated with the greatest velocity toward the anode and was followed by the slower moving alpha-globulins. $Beta_1$- globulin appeared as a thick dark band migrating behind the $alpha_3$- globulins in some samples and behind $alpha_4$-globulins in others. In the samples which contained 11 components, an additional subfraction was found in the beta- region; $beta_3$-globulin (Fig. 3). Fibrinogen entered the gel and migrated between beta- and $gamma_1$-globulins. The gamma-globulins were found in close proximity to where the protein sample was applied.

Figure 4 shows fractions which were resolved from rabbit plasma on filter-paper using a veronal buffer of pH 8.6. The fractions usually demonstrated were; albumin, alpha-, and beta-globulins, fibrinogen and gamma-globulin. However, the subfractions which appeared in the regions of alpha, beta, and gamma-globulins in the polyacrylamide gel were not observed on filter-paper.

In disc electrophoresis, using opossum plasma, 3 to 4 components were resolved. The separations were observed in order of decreasing mobilities; albumin, beta-, fibrinogen and gamma-globulin. The subfractions which appeared

in rabbit plasma were not observed in the plasma of the opossum (Fib. 5). However, on filter-paper alpha-globulin was observed between albumin and beta-globulin (Fig. 6). The content of albumin was very low which resulted in an albumin-globulin ratio below that of the rabbit.

CHAPTER V

DISCUSSION

Variations were observed in the number of separable proteins in rabbit plasma as compared with that of the opossums. These distinctive variations between different species were noted in the early studies of Moore ('45), Deutsch ('49), and Morris ('55). More recently, plasma protein differences between strains of the same species have been reported.

In disc electrophoresis, using opossum plasma, a complete isolation of components was not achieved. This may be due to components with similar mobilities which failed to separate when the protein mixture migrated through the gels.

The protein pattern using rabbit plasma revealed a higher percentage of albumin as compared with that of the alpha, beta, and gamma globulins. This was also observed in the studies of Svensson ('41), and Deutsch and Goodloe ('45). Fibrinogen was observed in all of the gels which was in agreement with the findings of McArthur ('62) who used starch-gel.

CHAPTER VI

SUMMARY AND CONCLUSIONS

1. The disc electrophoretic technique was found to be about 100 times more sensitive than the routine starch-gel or filter paper method. The pore size of the polyacrylamide gel can be adjusted.

2. The separations of a protein mixture can be accomplished in 30 minutes.

3. A complete isolation of opossum plasma in the polyacrylamide gel was not achieved.

4. Fibrinogen was observed in all of the samples between beta and gamma globulins.

5. The results of this study concur with those found by other investigators; that is, the number of plasma proteins observed in the plasma of rabbits was not found in the opossums.

LITERATURE CITED

Cooper, G. R. 1960 The Plasma Proteins. Ed. by F. W. Putman Vol. I, Academic Press, New York. Chap. III, 51-103.

Deutsch, H. and M. B. Goodloe 1945 An electrophoretic survey of various animal plasmas. J. Biol. Chem., 161: 1-20.

Fruton, J. S. and S. Simmonds 1958 General Biochemistry. John Wiley and Sons, Inc., New York, Chap. IV, 85-112.

Lewis, L. A. 1960 Electrophoresis in Physiology. Charles C. Thomas, U. S. A., Chap. III, 5-30.

McArthur, J. A. 1962 Isolation of rabbit plasma proteins of cold-ethanol fractionation and starch-gel electrophoresis. Thesis, Atlanta University.

Moore, D. H. 1945 Species differences in serum protein patterns. J. Biol. Chem., 161: 21-31.

Ornstein, L. 1962 Theory of Disc Electrophoresis. Cell Research Laboratory, The Mount Sinai Hospital.

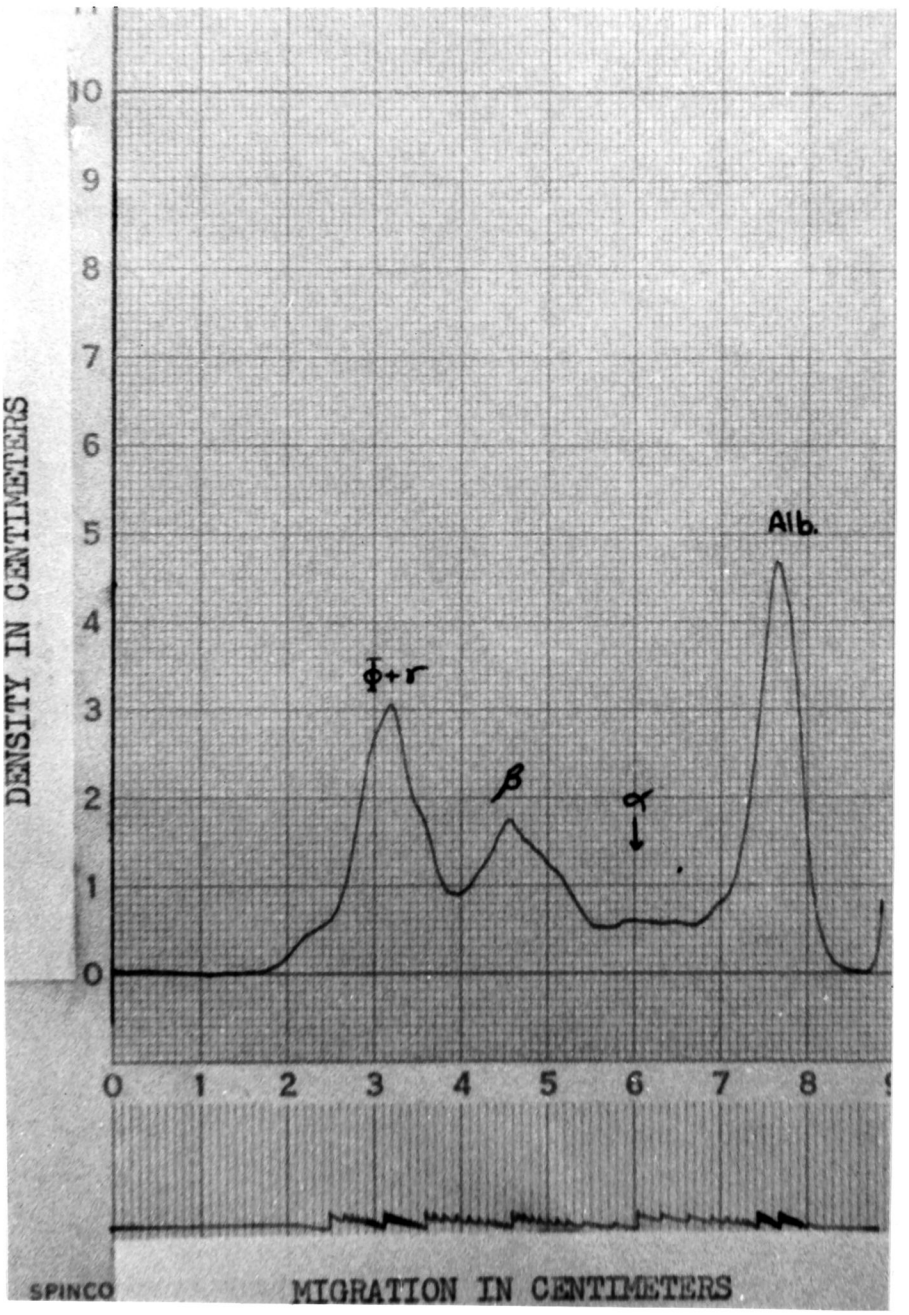
Poulik, M. D. and O. Smithies 1958 Comparison and combination of starch-gel and filter-paper electrophoretic method applied to human sera: Two-dimensional electrophoresis. Biochem. J., 68: 636-645.

Smithies, O. 1955 Zone electrophoresis in starch-gels: Group variations in the serum proteins of normal human adult. Biochem. J., 61: 629-641.

_____ 1959 An improved procedure for starch-gel electrophoresis: Further variations in the serum proteins of normal individuals. Biochem. J., 71: 585-587.

Svensson, H. 1941 Fractionation of serum with ammonium sulfate and water dialysis, studied by electrophoresis, J. Biol. Chem., 139: 805-825.

Tiselius, A. 1959 Electrophoresis, Ed. by M. Bier. Academic Press Inc., New York. Introd., XV-XX.


DENSITY IN CENTIMETERS
10
9
8
7
6
5
4
3
2
1
0
Alb.
Φ+r
β
α
0
1
2
3
4
5
6
7
8
SPINCO
MIGRATION IN CENTIMETERS